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# A Proximity-Dependent Biotin Labeling Based Screen For Protein Kinase A Anchoring Proteins Within Focal Adhesion Complexes

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# A PROXIMITY-DEPENDENT BIOTIN LABELING BASED SCREEN FOR PROTEIN KINASE A ANCHORING PROTEINS WITHIN FOCAL ADHESION COMPLEXES

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## ABSTRACT

Protein kinase A (PKA) regulates a diverse array of cellular activities including metabolism, differentiation, actomyosin contractility, and migration. The multifunctionality of this ubiquitous enzyme is achieved, in part, through subcellular targeting mediated by the A Kinase Anchoring Proteins (AKAP) family of proteins. AKAPs serve as scaffolding proteins that localize PKA to various cellular compartments and bring together specific targets and modulators of PKA activity.

The importance of spatially restricted PKA signaling is particularly apparent in the context of cell motility. It has been observed that both anchoring through AKAPs and the subsequent localized activation of PKA at the leading edge of migrating cells are required for directed migration in multiple cell types. Despite the significant body of evidence linking PKA to the regulation of cellular adhesion, contractility, and migration, the mechanisms governing the spatiotemporal control of PKA signaling during these activities is not fully understood. Focal adhesion complexes, which connect the actin cytoskeleton to the extracellular matrix and are thus intimately involved in the adhesive and contractile state of the cell, are attractive potential sites of PKA signaling. We have evidence indicating that PKA is active within these complexes, and that this activity impacts focal adhesion dynamics.

To address the question of how PKA may be recruited to adhesive complexes, we have developed a targeted screen to identify PKA interacting proteins within adhesive and cytoskeletal structures. This method utilizes proximity-dependent biotin labeling in combination with a focal adhesion purification preparation and downstream proteomic analysis. The results of this screen will be used to identify candidate AKAPs and will serve as the foundation for future inquiry into the complex role of PKA in the regulation of cell migration.

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## CHAPTER IV: SUPPLEMENTAL INFORMATION

## Abbreviations

**AKAP**- A-kinase anchoring protein

**BioID**- Proximity-dependent Biotin Identification

**cAMP**-cyclic adenosine monophosphate

**DPDPB**- 0.05 mM 1,4-di-[3'-(2'-pyridyldithio)-propionamido]butane

**DSP**- 0.5 mM dithiobis[succinimidyl propionate]

**DTT**- Dithiothreitol

**FA**- Focal Adhesion

**FACS**- Focal Adhesion and Cytoskeletal Enrichment

**LSB**- Laemmli Sample Buffer

**MS**- Mass Spectrometry

**NaDOC-** Sodium Deoxy Cholate

**PBS**- Phosphate Buffered Saline

**PKA**- Protein Kinase A

**SDS**- Sodium Dodecyl Sulfate

**SDS-PAGE**- SDS Polyacrylamide Gel Electrophoresis.

# CHAPTER I: BACKGROUND AND APPROACH

## 1.1 Introduction

The ability of cells to move within and throughout the various tissues of the body is a fundamental behavior that underlies essential physiological processes from development, to immune function, to tissue repair (Ridley et al., 2003). Migration and invasion also play a central role in the progression of certain diseases; notably the growth and metastasis of numerous different cancers (Clark and Vignjevic, 2015; Oudin and Weaver, 2016). The various intracellular events that must occur for successful migration require an exquisite degree of both spatial and temporal control. Cells must coordinate spatially distinct events including protrusion of the leading edge, reorientation of the cell body, and retraction of the trailing edge, as they actively probe and sense the environment through which they are moving (Reig et al., 2014; Ridley et al., 2003). Directed migration, or migration in response to environmental cues, requires integration of external signals to initiate both local and global changes within the cell (Petrie et al., 2009). Cells respond to extracellular signals such as soluble chemical factors and increases in matrix rigidity through processes termed ‘chemotaxis’ and ‘durotaxis’, respectively (Lo et al., 2000; Van Haastert and Devreotes, 2004), which are fundamentally important in both normal physiology and disease progression (de Oliveira et al., 2016; Oudin and Weaver, 2016; Petrie et al., 2009; Wozniak and Chen, 2009). These complex responses are achieved through multiple signaling pathways that work in concert to alter the adhesive and cytoskeletal organization in different regions of the cell (Devreotes and Horwitz, 2015; Ridley et al., 2003). Among the numerous signaling molecules associated with the

coordination of the processes that facilitate motility, the ubiquitous serine/threonine kinase, Protein Kinase A (PKA) has been found to play an important, but complex role in migration.

The primary focus of our lab for the last several years has been understanding how spatially restricted signaling of the cyclic adenosine monophosphate (cAMP) dependent kinase, PKA, regulates directed cell migration. PKA is active in nearly every compartment of the cell and regulates numerous vital cellular processes from metabolism to differentiation (Beebe, 1994; Taylor et al., 2005). In the absence of cAMP, PKA exists as a heterotetramer formed by two regulatory (R) subunits that each hold a catalytic (C) subunit in an inactive conformation; upon binding of cAMP to the dimerized R subunits, the enzymatically active C subunits are released and are free to phosphorylate nearby substrates (Johnson et al., 2001). The PKA holoenzyme exists in two forms; Type I and Type II, which are characterized by the inclusion of RIα/ß or RIIα/ß regulatory subunit isoforms, respectively (Fig. 1). The subcellular localization of PKA is achieved primarily through interactions between the PKA R subunits and a family of proteins known as the A-Kinase Anchoring Proteins (AKAPs) (Wong and Scott, 2004). The heterogeneous AKAP family possess the conserved A-Kinase Binding motif; which is characterized by a short (14-18 residue) amphipathic helix that sits in a hydrophobic pocket formed by the N-terminal Dimerization/Docking (D/D) domain of the R subunit dimer (Skroblin et al., 2010; Wong and Scott, 2004). Importantly, AKAPs form multiprotein signaling complexes by interacting with PKA substrates, other signaling molecules including GTPases and kinases, phosphodiesterases, and protein phosphatases (Fig. 1).

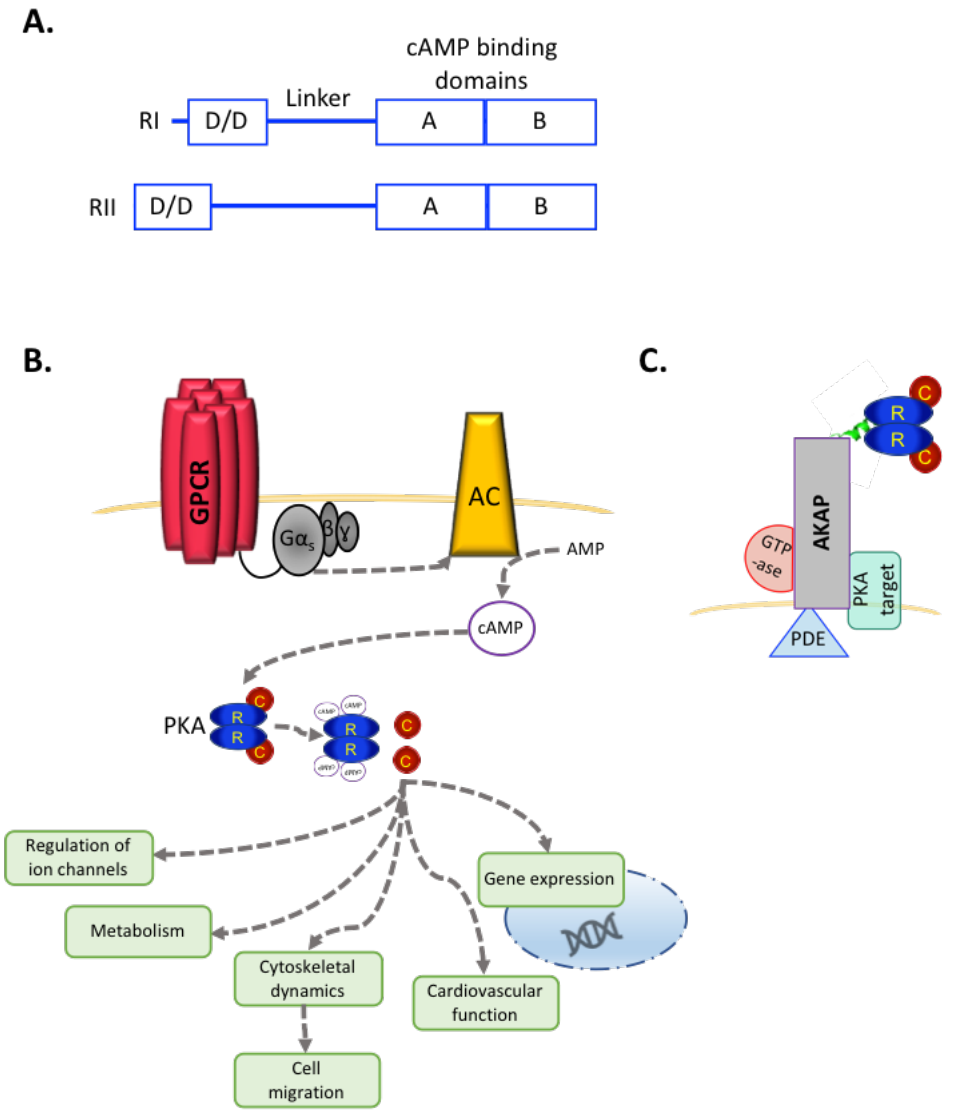


**Figure 1. Overview of PKA regulatory subunit isoforms, canonical activation pathway, and AKAP complex formation. (A)** Organization of the RI and RII isoforms of PKA including the N-terminal Dimerization/Docking domain (D/D), the linker region containing the catalytic subunit binding domain, and the two C-terminal cyclic-AMP binding domains (A and B). **(B)** Canonical activation of PKA downstream of G-Protein Coupled Receptor (GPCR) signaling. Activation of GPCR at the plasma membrane results in Gαs mediated Adenylate Cyclase (AC) activity and cAMP production. Binding of two cAMP molecules to each of the R-subunits leads to release of active catalytic subunits, which then phosphorylate proximal target proteins to regulate various cellular functions. **(C)** AKAPs bind the PKA regulatory subunit dimer, specific PKA targets, phosphodiesterases (PDE) and additional signaling molecules such as GTPases to form localized signaling complexes.

These AKAP mediated complexes serve to both localize PKA to different sub-cellular locations, as well as couple it to specific substrates or effectors. In addition to the ~50 members of the canonical AKAP family, several non-canonical anchoring interactions have also been identified. Notably, microtubules and actin have been shown to anchor Type I and Type II PKA respectively, through non-canonical binding mechanisms (Kurosu et al., 2009; Rivard et al., 2009). The vast majority of AKAPs interact specifically with RII subunits (e.g. WAVE1, AKAP-Lbc, gravin) although a few have been found to bind exclusively with RI subunits (e.g. smAKAP, Skip) (Gold et al., 2006), while others exhibit dual specificity (e.g. ezrin, AKAP10). PKA is known to have both positive and negative effects on adhesion and motility (Edin et al., 2001; Howe, 2004; Ydrenius et al., 1997), and the compartmentalization of PKA in critical to this complex regulatory role (Howe et al., 2005; Lim et al., 2008; Paulucci-Holthauzen et al., 2009).

The importance of the PKA/AKAP interaction in the regulation of cell migration is well established and the nature of this relationship has been examined in several cell types. For instance, localized PKA activity and anchoring through RII is required for chemotaxis in fibroblasts (Howe et al., 2005). Similarly, PKA activity at the leading edge of endothelial ovarian cancer (EOC) cells is required for migration and invasion, and while anchoring of both Type I and Type II PKA are necessary for EOC cell migration, leading edge PKA activity is mediated by RII AKAP interactions (McKenzie et al., 2011). Anchoring through canonical AKAP binding to AKAP-Lbc (Paulucci-Holthauzen et al., 2009) and non-canonical interactions with the α4 integrin (Lim et al., 2007) are known to be important for the localized PKA activity that promotes cell polarization and migration. Interestingly, in

addition to anchoring PKA to the leading edge, α4 integrins are also directly phosphorylated by PKA, and this regional regulation is essential for efficient α4ß1 mediated migration (Goldfinger et al., 2003).

The functional relationship between the adhesive state of the cell and PKA activation is well appreciated in the literature. Specifically, integrin mediated adhesion and detachment have both been shown to lead to rapid activation of PKA in different contexts (Howe and Juliano, 2000; Mercurio et al., 2001). More recently, it has been observed that local activation of PKA in response to increased matrix tension is also essential for durotaxis in EOC cells, although the specific AKAP interactions that govern this activity have not yet been determined (McKenzie, 2014). While there is evidence for reciprocal regulation of adhesion by PKA, as with α4 integrin, the mechanisms that govern this relationship are not well understood. The integrin associated adhesive complexes, focal adhesions, are attractive potential targets for PKA activity as the signals received and transmitted through focal adhesions help to coordinate the diverse cellular events required for motility (Kuo, 2013; Wozniak et al., 2004). Focal adhesions are dynamic structures that integrate signals received through integrin mediated interactions with the extracellular matrix and the actin cytoskeleton to regulate the adhesion, contractility, and migration. The hundreds of proteins that make up these complex structures include; the extracellular matrix binding integrins, actin binding proteins that connect the integrins to the cytoskeleton, adaptor and scaffolding proteins, along with numerous signaling molecules such as tyrosine and serine/threonine kinases, GTPases, and GTPase effectors (Burridge and Chrzanowska-Wodnicka, 1996; Kanchanawong et al., 2010). Proteomic analysis of the

integrin “adhesome” has revealed that several well established PKA interacting proteins reside within adhesion complexes (Horton et al., 2015). Tyrosine kinases Src and Fyn are core modulators of focal adhesion dynamics and known targets of PKA phosphorylation. Other notable, intrinsic focal adhesion proteins that are PKA substrates include the small GTPase RhoA, the actin binding proteins VASP and filamin a, and α4 integrin. Additionally, sequence analysis of the adhesome reveals several components that contain the PKA consensus sequence, suggesting that novel PKA substrates may be found within adhesive complexes (A. K. Howe, unpublished). While direct phosphorylation of these substrates in the context of adhesive structures has not been demonstrated, the PKA mediated regulation of many of these substrates has been shown to impact adhesion and migration (Doppler and Storz, 2013; Newell-Litwa and Horwitz, 2011; Schmitt and Stork, 2002; Yeo et al., 2011). PDE4, a member of the phosphodiesterase family of enzymes, has also been identified as an integral focal adhesion component. Phosphodiesterases hydrolyze cAMP and thus act as the primary modulators of PKA activity. The presence of PDE4 indicates that a mechanism for inactivation of PKA exists within these structures. Finally, a handful of AKAPs (both canonical and non-canonical) including ezrin, AKAP 9, AKAP2, and actin, are also associated with focal adhesion complexes. Taken together, these previous proteomic analyses establish a theoretical framework for PKA signaling within focal adhesions.

The Howe Laboratory has observed previously that both inhibition of PKA activity and disruption of anchoring through AKAPs significantly impacts adhesion morphology and dynamics in EOC cells (McKenzie, unpublished). Specifically, cells expressing

peptide inhibitors of either PKA activity or anchoring produced adhesions that were smaller in size, exhibited slower rates of assembly, and longer overall lifetimes. Additionally, disruption of RII anchoring led to a decrease in the disassembly rate of focal adhesions. We are currently in the process of repeating these studies in the rat embryonic fibroblast cell line (REF52) and our preliminary data indicate similar morphological effects (H. Naughton & K. Medeiros, unpublished).

While the circumstantial evidence is significant, there are very few studies that directly assess PKA activity within focal adhesion complexes. To address this, we generated the Paxillin-linked ratio-metric A-kinase sensor (Pax-RAKS), a focal adhesion targeted biosensor that allows us to monitor PKA activity within adhesive structures (Fig. 2). The single-fluorophore PKA biosensor, GAKdy, which utilizes a conformationally sensitive GFP variant was used as the basis for the Pax-RAKS sensor (Bonnot et al., 2014). The adaptor protein Paxillin was fused to the N-terminus of GAKdy to facilitate targeting of the sensor to focal adhesion complexes. An mCherry tag was inserted ahead of paxillin to serve as an internal control for fluctuations in fluorescence intensity as a result of protein flux within focal adhesions. The ratio of GFP to mCherry intensity is thus used to detect changes in PKA activity within a given region of the cell. We measured discrete changes in the biosensor intensity within focal adhesions that varied in magnitude, duration, and area in cells monitored by either total internal reflection microscopy (TIRF) or super-resolution confocal microscopy (Fig. 2B-D; H. Naughton and A. K. Howe, unpublished). Importantly, similar fluctuations were not detected in the phospho-resistant PAX-RAKS T/A biosensor, indicating that the changes measured with the wild-type sensor were due to

phosphorylation by PKA (Fig. 2E). These data, while preliminary in nature, indicate that there is spatially and temporally dynamic activation of PKA within focal adhesion complexes, and support the notion that PKA associates with the adhesive and cytoskeletal complexes.

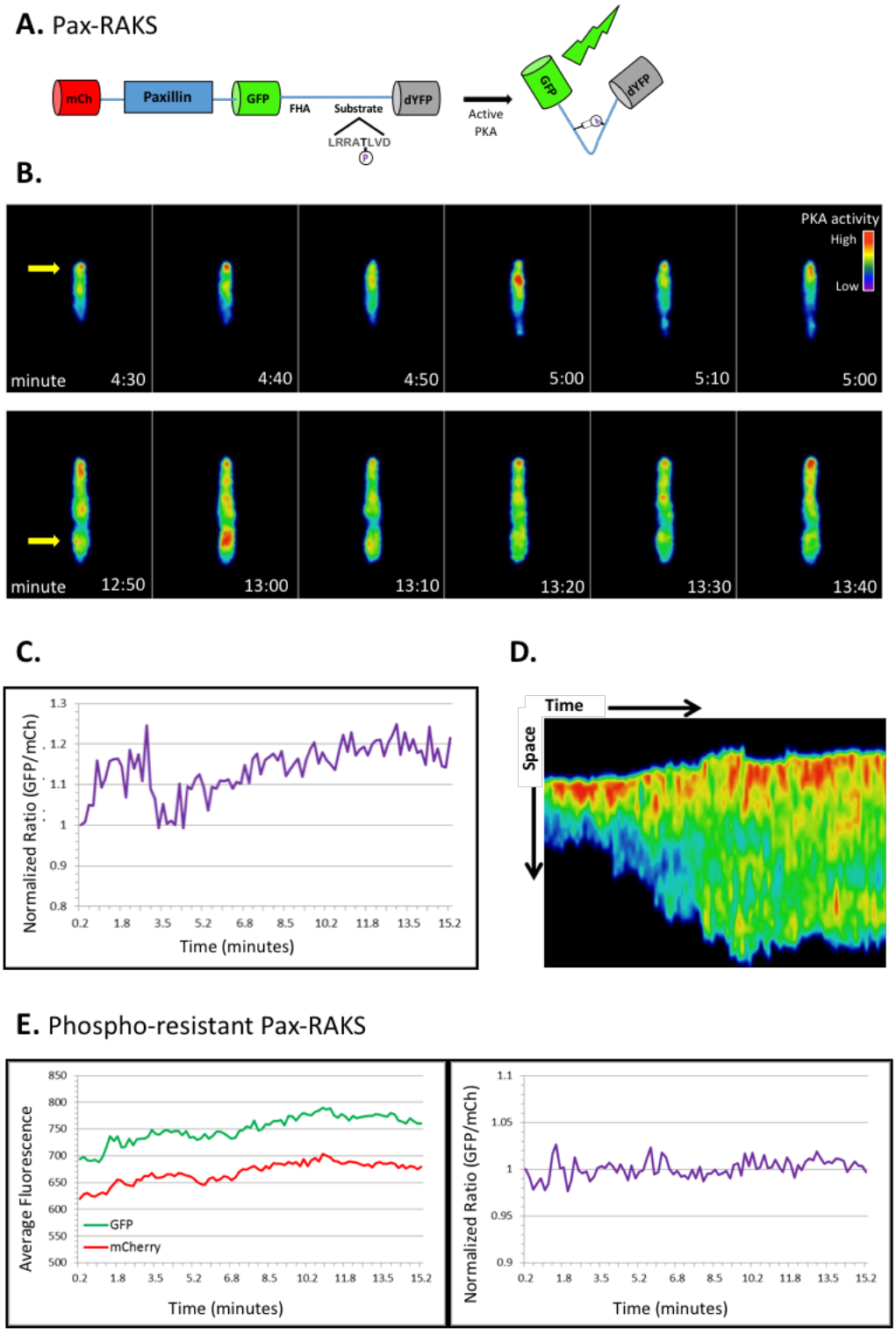


**Figure 2. Dynamic, localized changes in PKA activity within focal adhesions detected using a targeted, single-chain biosensor, Pax-RAKS.**

**Figure 2. Dynamic, localized changes in PKA activity within focal adhesions detected using a targeted, single-chain biosensor, Pax-RAKS**. (**A**) Pax-RAKS was generated by inserting mCherry (mCh) tagged paxillin at the N-terminus of the GAkdY biosensor. GAkdY is composed of a conformationally sensitive GFP fluorophore and a dark YFP molecule (dYFP) connected by a PKA substrate recognition sequence (LRRATLVD) and a fork head associated (FHA) domain. Phosphorylation of the Threonine residue in the substrate domain promotes binding to the FHA domain. The subsequent conformational change results in an increase in GFP intensity, which is reported here as an increase in the Pax-RAKS ratio. (**B**) Dynamic changes in PKA within focal adhesions were visualized using custom Image J macros that extract, register, and align an individual adhesion from a time-lapse series. The Pax-RAKS ratio is then calculated for each frame (acquisition every 10 seconds) and the resulting stack is pseudo-colored such that warmer colors represent areas of high PKA activity. The arrow in the top panel highlights a region of high PKA activity in the distal portion of the adhesion. The arrow in the bottom panel highlights a region of high PKA activity in the proximal end of the same adhesion. (**C**) The overall Pax-RAKS ratio (GFP/mCh) of the adhesion shown in B over the course of the acquisition. (**D**) A linescan through a linear region of the pseudo-colored series of the same adhesion. (**E**) The average intensity of the GFP and mCh fluorophores (left) and the normalized Pax-RAKS ratio (right) measured in an individual focal adhesion from a cell expressing the phospho-resistant Pax-RAKS(T/A) mutant biosensor.

## 1.2 Rationale

In summary, there are strong functional connections between PKA and focal adhesions: many focal adhesion proteins have known or putative PKA phosphorylation sites; disruption of normal PKA activity alters focal adhesion dynamics; and a targeted biosensor suggests that PKA activity is dynamically regulated within individual focal adhesions. Based on these functional connections, we hypothesize a physical connection; namely, that there exists one or more focal adhesion AKAPs that coordinate these events. Here we describe a proteomics-based screen for novel AKAP interactions within adhesive structures. This method utilizes proximity-dependent biotin identification (BioID) in combination with a focal adhesion purification method that has been used previously for quantitative adhesome analysis. We aim to utilize the procedure described here to identify interactions between PKA and focal adhesion proteins, and ultimately generate a list of candidate AKAPs that can be used to guide our future efforts to elucidate the complex role of PKA in the regulation of cell migration.

## 1.3 Overview of Procedure

The following procedure describes a proteomics-based screen for novel PKA interacting proteins, specifically anchoring proteins, within the adhesive and cytoskeletal structures of adherent cells. This protocol utilizes proximity-dependent biotin labeling (Roux et al., 2012) in combination with a stringent focal adhesion isolation preparation (Schiller et al., 2011) to isolate biotin-labeled proteins that are associated with adhesive complexes (Fig. 3). Targeted labeling of PKA interacting proteins is achieved by fusion of a promiscuous biotin ligase to the regulatory subunit of PKA. For this experiment we opted

to use the modified biotin ligase from *Aquifex aeolicus*, known as BioID2 (with 25 nm flexible linker) (Kim et al., 2016). For ease of communication the focal adhesion and cytoskeletal enrichment protocol is referred to as the FACS preparation from this point forward. Briefly, rat embryonic fibroblasts (REF52) transiently expressing one of the BioID2/R-subunit fusions and non-transfected control cells are incubated with biotin overnight to allow for biotinylation of PKA-proximal proteins (Fig. 3B[1]). To isolate focal adhesion and cytoskeletal structures, cells are then treated with a cell permeable crosslinking solution to stabilize protein-protein interactions. Cells are lysed with an ionic detergent containing buffer and the dish is then subjected to a high-pressure fluid stream which removes residual, membrane, cytoplasmic and nuclear material, while preserving much of the cytoskeletal and adhesive framework (Fig. 3B[2]). A buffer containing the reducing agent dithiothreitol (DTT) is then used to reduce the chemical crosslinks and elute the adhesion associated proteins. The focal adhesion fraction is collected and purified by acetone precipitation. The biotin labeled proteins in the purified sample are isolated with streptavidin-coated, magnetic beads prior to analysis by mass spectrometry (MS) (Fig. 3B[3]). The proteins identified in the BiID2/R-subunit fusion samples can then be compared to those found in the non-transfected control samples and those proteins unique to the BioID2 samples can be can be individually assessed for AKAP characteristics.

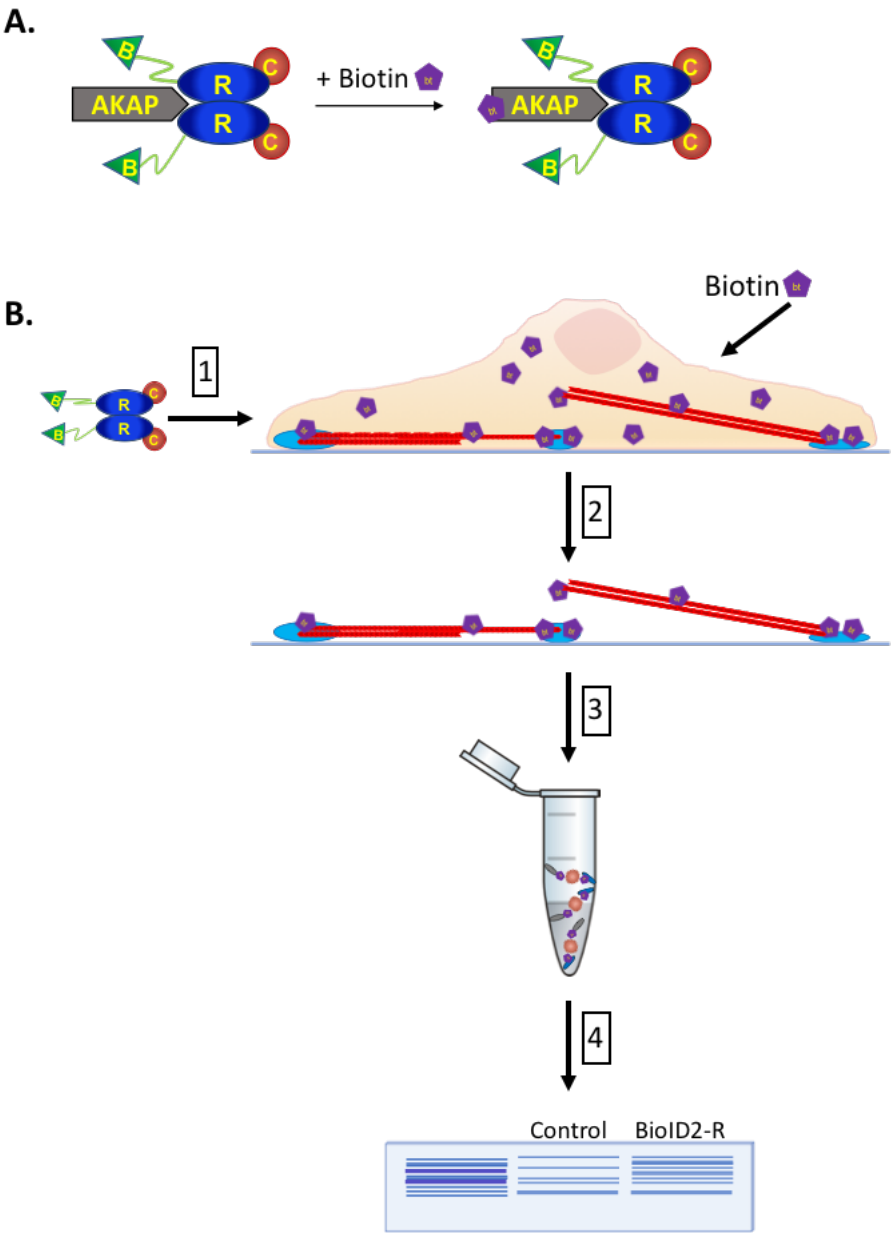


**Figure 3. Summary of the approach for biotin labeling and purification of PKA-interacting proteins within adhesive and cytoskeletal structures.**

**Figure 3. Summary of the approach for biotin labeling and purification of PKA-interacting proteins within adhesive and cytoskeletal structures.** (**A**) Targeted biotin labeling of PKA-proximal proteins using recombinant BioID2/R-subunit fusion proteins. PKA regulatory subunits (blue oval, 'R') with attached BioID2 biotin ligase (green triangle, 'B') bind to interacting partners including the catalytic subunits (red circle, 'C') and A kinase anchoring proteins (gray arrow, 'AKAP'). Upon addition of biotin, BioID2/R-subunit biotinylates proximal proteins. (**B**) Schematic of protocol for isolation of biotin-labeled, focal adhesion and cytoskeletal PKA interacting proteins by FACS preparation. [1] Cells are transfected with BioID2/R-subunit constructs and incubated with excess biotin. [2] Cytoskeletal and adhesion complexes are stabilized by chemical crosslinking, cells are lysed, and residual, non-adherent cellular material is removed by high-pressure wash. Focal adhesions and cytoskeletal structures remain intact. [3] The focal adhesion fraction is collected, purified by acetone precipitation, and the biotin-labeled proteins within this fraction are isolated by affinity capture using streptavidin-conjugated magnetic beads. [4] The purified focal adhesion fractions from BioID2/R-subunit expressing cells, and non-transfected control cells are minimally separated by SDS-PAGE and stained with colloidal Coomassie blue in preparation for proteomic analysis.

## 1.4 Applications

The complexity and dynamic nature of focal adhesions makes studying the individual protein-protein interactions within these complexes fairly challenging. When considering a ubiquitous kinase, such as PKA, which is active in nearly every compartment of the cell - the identification of interactions that occur specifically within focal adhesion complexes becomes even more complex. To our knowledge, this is the first-time proximity-dependent biotin labeling has been used to screen for PKA interacting proteins. The approach described here allows us to label proteins that interact with PKA and then isolate those proteins that are both labeled and found within adhesive structures. Through insertion of the BioID2 ligase to the regulatory subunit of PKA, we aim to enrich anchoring proteins which interact specifically with the regulatory subunit dimer. The proteomics data from BioID2-RII expressing cells will be compared to data from non-transfected cells, and proteins found specifically in the BioID2 data set will be vetted and scored for AKAP potential based on the strength of the peptide match, the presence of a helical AKAP consensus motif, as well as pathway analysis (e.g. connections with potential PKA substrates). Those proteins that present as the strongest candidates can then be more thoroughly assessed for PKA binding and AKAP function through biochemical analyses.

While the targeted biotin labeling afforded by the BioID2 fusion construct does ensure labeling of proteins that are in close proximity to PKA, a physical interaction with PKA is not required for biotin labeling to occur. Therefore, it is possible, and perhaps likely that PKA substrates, and other components of PKA/AKAP signaling complexes (e.g. phosphatases and other kinases) associated with focal adhesions may also be labeled by

this approach. As such, proteomic hits will also be assessed for substrate characteristics (i.e. PKA phosphorylation consensus site).

Importantly, this method can be modified to probe specifically for PKA substrates within focal adhesions by integrating the biotin ligase to the catalytic subunit. Phosphoproteomic analysis of these samples would aid in verification of PKA targets within adhesive complexes. Furthermore, this approach can be utilized to screen for other novel protein-protein interactions within focal adhesions.

### 1.5 Limitations

The use of the BioID2 enzyme with a flexible linker extends the labeling radius to greater than 35 nm (Kim et. al 2016). This increases the probability that BioID2 will label anchoring proteins associated with the R-subunit, but also makes it more likely that proximal proteins not directly associated with PKA will be tagged. It is important to recognize that under these circumstances, biotinylation does not signify direct interaction. Each candidate AKAP will need to be individually assessed for PKA binding capacity to confirm specific interactions.

Given the position of the AKAP recognition pocket at the N-terminus of both the type I and type II R-subunit dimers, it stands to reason that positioning the BioID2 at the N-terminus would provide the best opportunity for labeling AKAPs. However, although the 13 GGGGS repeat linker provides substantial separation (~25 nm) between the terminal residues that form the AKAP binding pocket, it is possible that the presence of the BioID2 enzyme at the N-terminus of the regulatory subunit could disrupt the access to, or conformation of the hydrophobic AKAP binding site. As N-terminal PKA regulatory

subunit fusions are not common in the literature, we cannot predict if anchoring will be impacted, and characterization of the AKAP binding capacity of BioID2/R-subunit fusion proteins is necessary. Potential interference with AKAP binding could likely be avoided by positioning BioID2 at the C-terminus, as evidenced by the fact that C-terminal fluorescent tags have been used without any reported anchoring deficits (Manni et al., 2008). However, it is unclear if the labeling radius of BioID2 with the added linker will be sufficient to effectively biotinylate proteins that interact specifically with the N-terminus of the regulatory subunit. In light of these considerations, the AKAP binding *and* labeling capacity of both N and C-terminal BioID2 fusions need to be assessed.

### 1.6 Anticipated Results

We have established a protocol that enables us to selectively label proteins that interact with (or are in close proximity to) the regulatory subunits of PKA, and then enrich for those biotinylated proteins that are associated with focal adhesion complexes. We anticipate that proteomic analysis of the purified focal adhesion fraction will yield a data set that is predominantly composed of known cytoskeletal and adhesion associated proteins, although it is possible that a small number of cytosolic proteins not excluded by the FACS preparation may be identified as well. If a substantial portion of the proteins identified are not associated with adhesive structures (e.g. mitochondrial or nuclear proteins), it will be necessary to re-asses the stringency of the FACS preparation. Though this procedure has been used previously for quantitative proteomic analysis of focal adhesions, it is possible that it will require further optimization to accommodate cell type or equipment differences.

It is possible that control samples not expressing BioID2 may still contain proteins that are pulled down by the streptavidin-beads. This may be a result of endogenous biotin ligase activity, or to a lesser extent, non-specific binding to the streptavidin beads. While we do not expect there to be significant overlap in the peptides identified in BioID2/R-subunit and control samples, any proteins found in both data sets will be excluded from further analysis. It is important to note that additional control conditions can, and likely should be utilized for this primary level of assessment. Comparison to data sets generated from the biotin-labeled FA fraction of cells expressing the non-targeted BioID2 enzyme, or under conditions in which AKAP binding is disrupted, would further aid in the identification of candidate AKAPs.

Through previous efforts to identify novel AKAP interactions, we have developed an algorithm that can be used to objectively score identified proteins for AKAP potential (Director, 2014 ). With minor modifications, this algorithm could be applied to our data set to evaluate potential AKAPs based on the following criteria: mass spec strength, number of peptides identified, biological relevance, presence of AKAP consensus motif characterized by the conserved pattern of hydrophobic residues; [AVLISE]-XX-[AVLIF]-[AVLI]-XX-[AVLI]-[AVLIF]-XX-[AVLISE], where 'X' represents any amino acid (Skroblin et al., 2010), and helical projection of AKAP consensus site. While we expect that use of this algorithm will implicate some of the more well-known AKAPs that are associated with the cytoskeleton and focal adhesions such as WAVE1 and ezrin, we also anticipate that previously unrecognized anchoring proteins with potential AKAP characteristics may be identified. Based on previous screens we expect the actin binding

proteins, filamin-A and talin may be identified as high scoring candidate AKAPs through this screen (Director, 2014 ). While the scoring algorithm is useful for identifying canonical AKAPs, it is possible that some of the identified proteins interact with PKA through non-canonical mechanisms. Proteins that have a high degree of biological relevance (i.e. cytoskeleton interacting proteins, core focal adhesion components) *and* have connections to known PKA signaling networks, should be considered even in the absence of an AKAP consensus sequence.

This screen is designed to provide a list of *candidate* PKA interacting proteins within focal adhesions. All putative anchoring proteins identified through this protocol must be verified by additional biochemical testing. We aim to use this screen as the foundation for our continued exploration of the complex regulation of cellular motility by PKA and anticipate that these data will be used to identify novel signaling complexes.

# CHAPTER II: EXPERIMENTAL DESIGN

## 2.1 Generation of BioID2-Regulatory subunit fusion proteins

There is not yet definitive evidence for which PKA isoform is primarily responsible for the activity associated with focal adhesions. Thus, for our initial screen we designed BioID2 fusion constructs with both Type 1α (RIα) and Type IIα (RIIα) regulatory subunits. BioID2 was selected for this application not only because it is more efficient than the original BirA* enzyme used for proximity-dependent biotin labeling, but the smaller size (23 kDa vs. 35 kDa) is less disruptive to the trafficking of fusion proteins to various subcellular compartments (Kim et al. 2016). Additionally, we utilized a 13 GGGGS repeat linker added to either the N or C terminus of BioID2 which increases the biotinylation radius of the ligase without affecting its activity (Kim et al. 2016). Given the location of the AKAP binding site at the N-terminus of the PKA R-subunit dimer, it stands to reason that an N-terminal BioID2 fusion would be optimal for labeling anchoring proteins. However, as mentioned previously, it is possible that the presence of the ligase at the N-terminus could disrupt either the AKAP binding pocket or the adjacent dimerization domain. Therefore, we designed both N and C-terminal BioID fusions for each regulatory subunit to account for this possibility.

Gibson assembly was used to insert mouse RIα or RIIα after the C-terminal linker of myc-BioID2-13X Linker (Addgene plasmid # 92308) to generate the N-terminal BioID2 fusions: BioID2-RIα and BioID2-RIIα (Fig. 4A). Similarly, each subunit was inserted ahead of the N-terminal linker of 13X Linker-BioID2-HA (Addgene plasmid #80899) to generate the C-terminal BioID2 fusions: RIα-BioID2 and RIIα-BioID2 (Fig. 4B). Clones

were screened by BamHI diagnostic digest, and positive clones were confirmed by sequencing (see Supplemental Methods).

*2.1.1 AKAP binding mutants*

In anticipation of future studies in which more stringent controls will be required to verify putative AKAP interactions, we created BioID2/R-subunit AKAP binding mutant constructs. Substitution of key residues in the D/D domain of the RII subunit have been shown to disrupt the binding of AKAPs with the RII dimer. Specifically, RII-AKAP binding can be inhibited by substitution of the isoleucine residues at positions 3 and 5 with hydrophilic amino acids, including serine (Hausken et al., 1996). Likewise, substitution with histidine in place of the cysteine residue at position 37 of the RI subunit disrupts RI-AKAP interactions (Banky et al., 1998). Site-directed mutagenesis was used to generate RIIα-I3S, I5S and Riα-C37H N and C terminal BioID2 fusions (see Supplemental Methods).

## 2.2 Expression of BioID2 fusion proteins in REF52 cells

The REF52 cell line was selected as an ideal candidate for the present study due to their ability to form numerous, prominent focal adhesions on standard plastic tissue culture dishes, as well as the ease of their culture and transfection. As the BioID2 plasmids used as the backbone of the fusion constructs contain a Neomycin resistance cassette, we endeavored to establish stable cells lines with each of the four BioID constructs. The use of stable cell lines in which all of the cells express the BioID2 fusion proteins would greatly increase the sensitivity of the screen. The stringent nature of the focal adhesion preparation

is ideal for removal of cellular material that is not associated with adhesions. However, it is likely that some percentage of focal adhesion proteins are lost during this step as well. Thus, maximizing the output of this process through the use of stable cell lines would increase the recovery of labeled proteins and facilitate identification of low-abundance proteins of interest in the downstream mass spectrometry analysis. Though we were successful in generating stable G418-resistant REF52 cell lines with each of the four constructs, we found that in all cases, the cells did not express full length fusion protein (Fig. S1). In light of this, we decided to proceed using transfection with FuGENE 6 reagent to transiently express fusion proteins until we are able to successfully generate stably expressing cell lines. With plasmids of similar size (~7.5 kb), we are typically able to achieve transfection efficiencies between 40-60% in REF52 cells using FuGENE 6. The majority of data presented here were generated with transiently expressing REF52 cells, however, transiently expressing HEK 293 cells were utilized for certain preliminary experiments in which their superior ability to express recombinant proteins was valuable.

## 2.3 Characterization of BioID2 fusion proteins

### *2.3.1. Detection of full length BioID2/R-subunit fusion proteins*

Western blot analysis using antibodies against myc (for BioID2-RIα and BioID2-RIIα) or HA (RIα-BioID2 and RIIα-BioID2) epitope tags was performed to confirm the expression of the BioID2/R-subunit fusion proteins in REF52 cells. We were able to detect bands corresponding to the predicted molecular weights of each of the full-length fusion proteins in lysates from transiently expressing REF52 cells (Fig. 4C-D). In addition to the full-length protein, one or more lower molecular weight species were detected in each of

the immunoblots, particularly in the HA-tagged, C-terminal constructs. These lower molecular species may be a result of proteolytic degradation, the nature of which we do not understand at this time. Despite this, all fusion proteins appear to be readily expressed by REF52 cells. The overexpression of RIIα-BioID2 and BioID2-RIIα was assessed by immunoblot using an antibody against the RIIα subunit. The full-length proteins are present at levels 2.6 times that of endogenous RIIα (Fig. 4E).

### *2.3.2. Confirmation of biotin ligase activity*

To assess the biotin ligase activity of each fusion protein, transiently expressing REF52 cells and non-transfected control cells were incubated with 50 μM biotin for 20 hours prior to lysis and biotin labeling was detected by streptavidin-HRP immunoblot (see Supplemental Methods). All four BioID2/R-subunit fusion proteins were found to be enzymatically active with subtle differences in both the extent of biotinylation and the distribution (i.e. molecular weights) of labeled proteins (Fig. 4F-G).

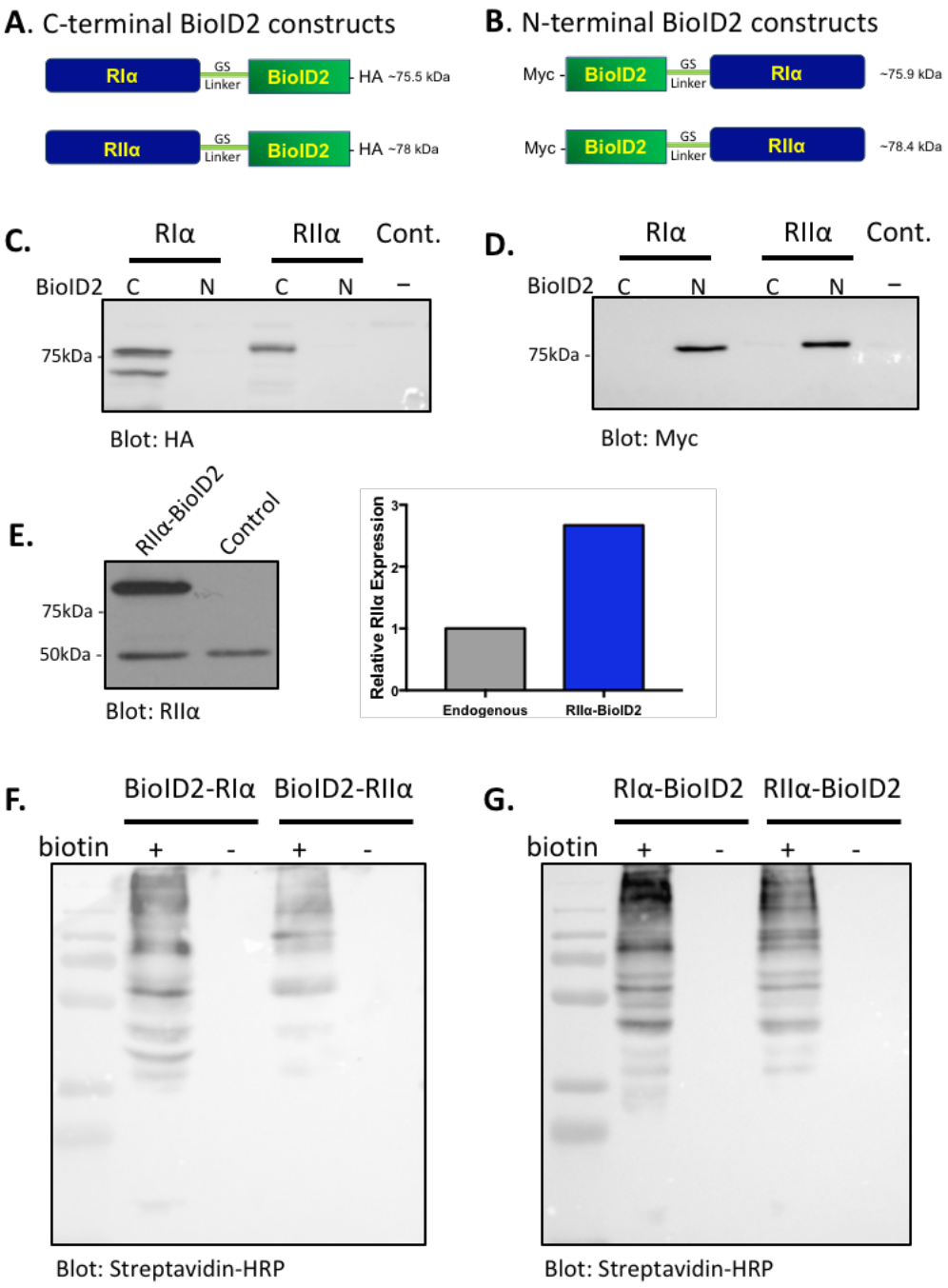


**Figure 4. Characterization of the expression and ligase activity of BioID2/R-subunit fusion proteins.**

**Figure 4. Characterization of the expression and ligase activity of BioID2/R-subunit fusion proteins.** (**A**) HA-tagged BioID2 with a N-terminal 13x GGGGS linker was inserted at the C-terminus of the RIα and RIIα subunits to make RIα-BioID2 and RIIα-BioID2 respectively. (**B**) Myc-tagged BioID2 with a C-terminal 13x GGGGS was inserted at the N-terminus of the RIα and RIIα subunits to make BioID2-RIα and BioID2-RIIα respectively. The predicted molecular weight is indicated at the left of each construct. (**C-D**) Lysates from non-transfected (Cont.) or transiently expressing REF52 cells were probed first with antibody against the myc epitope tag (**D**) and then with antibody against the HA tag following quenching and re-blocking of the membrane (**C**). Bands corresponding to the predicted molecular weights of both the RIα and RIIα C-terminal BioID2 fusion proteins are visible in **C**. Bands corresponding to the predicted molecular weights of both the RIα and RIIα N-terminal BioID2 fusion proteins are visible in **D**. (**E**) The overexpression of the BioID2/R-subunit fusions were evaluated in lysates from HEK293 cells transiently expressing RIIα-BioID2. Western blot analysis using an antibody that recognizes the RII subunit reveals a band corresponding to endogenous RII subunit at ~55 kDa in both transfected and non-transfected (Control) cells. The band at ~78 kDa in the RIIα-BioID2 lysate corresponds to the full-length fusion protein which is ~2.6 fold more abundant than the endogenous R subunit in this sample. The relative expression of RIIα-BioID2 to endogenous RIIα was determined by densitometry using the FIJI Gel Analyzer function (Image J). Full blot shown in Fig. S2. (**F-G**) Lysates from transiently expressing REF52 cultured in the presence or absence of biotin were probed with streptavidin-HRP to assess the biotin ligase activity of each of the BioID2/R-subunit fusions.

*2.3.3. Detection of AKAP79 following affinity purification of biotin-labeled proteins.*

Following confirmation of both BioID2/R-subunit expression and ligase activity, we evaluated the ability of the RIIα fusions to label the abundant, RII specific, membrane localized anchoring protein, AKAP79. To assess biotin labeling of AKAP79, we transfected HEK293 cells with BioID2-RIIα, RIIα-BioID2, or the I3S, I5S AKAP binding mutant versions of each construct and incubated with 50 μM biotin for 20 hours (see Supplemental Methods). Biotin labeled proteins were purified from the lysates of control and BioID2/RII-subunit expressing cells using streptavidin conjugated magnetic beads (see sections, *3.2.6.* and Supplemental Methods). The eluates from the streptavidin-bead affinity purification where assessed by western blot using an AKAP79 specific antibody. AKAP79 was detected in the eluate of both the wild type and I3S, I5S mutant samples, but was not present in the non-transfected control sample (Fig. 5B). This suggests, albeit indirectly, that the AKAP79 present in the eluate was biotin-labeled, and not solely the result of non-specific binding to the streptavidin-coated beads. Although AKAP79 appears to have been biotin-labeled in cells expressing both the wild type and mutant constructs, there does appear to be less biotinylated AKAP79 present in the eluate from the I3S, I5S mutant samples, despite the fact that AKAP79 expression in the whole cell extract was roughly equivalent (Fig. 5A). Streptavidin-HRP blotting of the same samples revealed that the overall biotin ligase activity of BioID2-RIIα was lower than that of the other fusion proteins (Fig. 5B) which may account for the apparent inferior labeling of AKAP79 as compared to RIIα-BioID2.

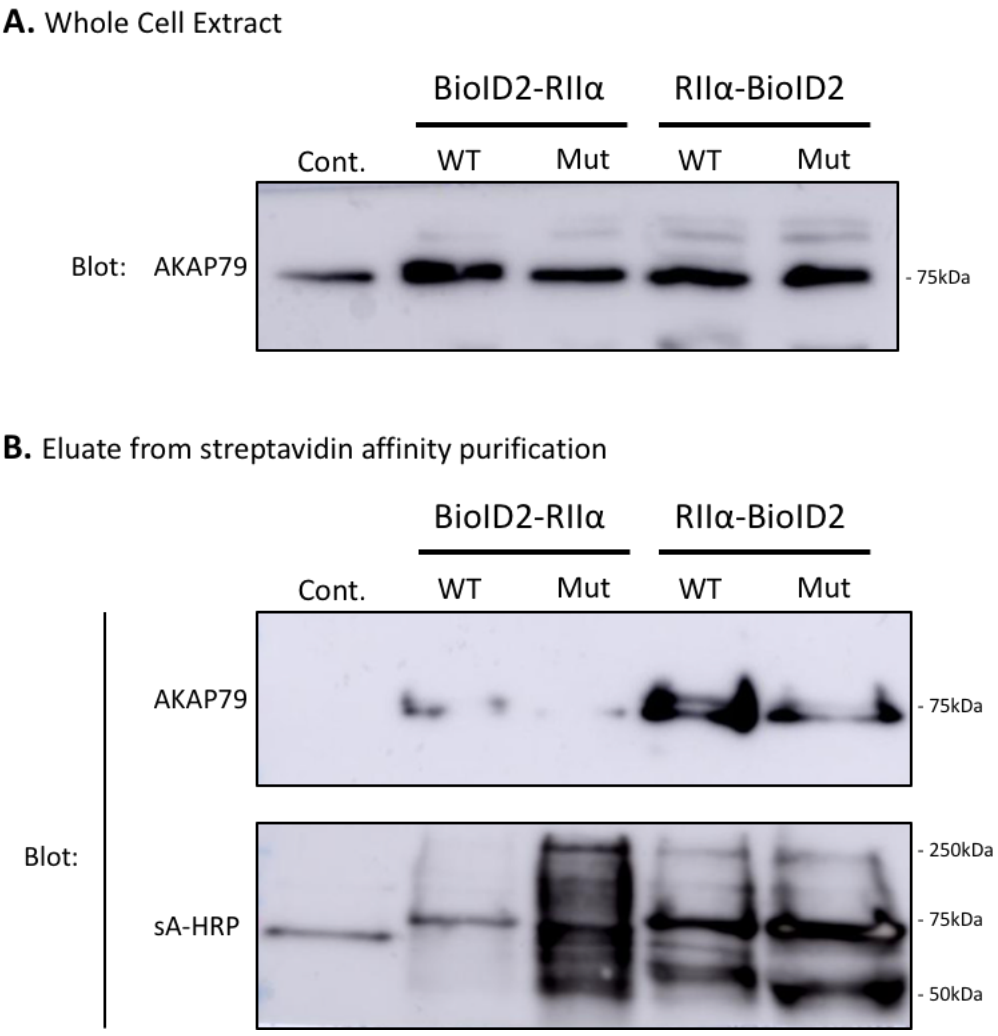


**Figure 5. AKAP79 detected in eluate following streptavidin-bead affinity purification.** Non-transfected (Cont.) and transiently expressing HEK 293 cells were incubated with 50 μM biotin overnight and lysed in modified RIPA buffer. (**A**) A portion of the whole cell extract was separated by SDS-PAGE and immunoblotted with an antibody against AKAP79. (**B**) 1.5 mg of whole cell extract was purified with streptavidin-coated magnetic beads and biotin labeled proteins were eluted by boiling in 1x LSB, 4 mM biotin for 3 minutes. The eluate was separated by SDS-PAGE and immunoblotted with an AKAP79 antibody (top). The membrane was treated with hydrogen peroxide and sodium azide to quench the HRP enzyme, and then incubated with streptavidin-HRP (sA-HRP) to detect the biotinylated proteins present in the eluate (bottom).

The BioID2-RIIα fusion did not show reduced biotin ligase activity in other experiments, and we do not know what caused the apparent discrepancy in this instance. It is not clear at this time why labeled AKAP79 was detected in the AKAP binding mutant samples, however, the apparent decrease of AKAP79 in these samples when compared to the wild type constructs could indicate impaired AKAP binding. This experiment should be repeated to confirm these observations. In addition, it will be important to confirm the expected effect of the I3,5S mutation by co-IP of the labeled constructs, irrespective of biotinylation.

### 2.4 Focal Adhesion and Cytoskeletal Enrichment (FACS) Preparation

To enrich for cytoskeletal and adhesive structures, we followed the focal adhesion purification procedures developed by Schiller et. al (2011) for the quantitative proteomic analysis of the integrin adhesome. In general, the preparation was carried out according to the published procedure, however, certain parameters were optimized to accommodate the equipment available, as well as the sample requirements for MS analysis by the University of Vermont Proteomics Facility. Briefly, cells are treated with two cell-permeable chemical crosslinkers; dithiobis[succinimidyl propionate] (DSP) and 1,4-di-[3'-(2'-pyridyldithio)-propionamido]butane (DPDPB) which crosslink lysine and cysteine residues, respectively. Crosslinking stabilizes protein complexes, including the integrin associated adhesion complexes. The crosslinking reaction is quenched by the addition of 1M TRIS-HCl pH 7.5 and the cells are then lysed in radioimmunoprecipitation assay (RIPA) buffer. The cell lysate is collected and retained for future use (this fraction is referred to as the 'whole cell lysate' (WCL). The plate is then subjected to a high-pressure fluid stream of complete PBS

which effectively removes all residual cellular material that is not firmly attached to the dish. After clearing of the dish is visually confirmed via brightfield microscopy, the stabilized, integrin associated complexes remaining on the dish are reduced by addition of a DTT containing buffer and collected by manual scraping. Finally, the proteins in the focal adhesion (FA) fraction are purified by acetone precipitation and resuspended in non-reducing Laemmli sample buffer.

## 2.5 FACS preparation with REF52 cells

### *2.5.1. Assessment of WCL and FA fractions by SDS-PAGE*

The protein profiles of the whole cell lysate WCL and FA fractions from initial FACS preparations performed with REF52 cells were visualized by Coomassie stain following separation by SDS-PAGE (Fig. 6A). When the WCL and FA fractions from our REF52 cell samples are compared side-by-side, the two fractions appear to have very different compositions based on the apparent molecular weights of the bands visible in each sample. As the sample buffer used to resuspend the FA fraction is not compatible with standard assays for protein concentration determination, Densitometry analysis of the Coomassie stained gels can be used to determine the relative protein concentration in the FA fraction (see Supplemental Methods; Table S 3).

*2.5.2. Detection of focal adhesion and cytosolic proteins in WCL and FA fractions*

Western blot analysis of the two fractions show that the focal adhesion proteins vinculin and talin are present in both the WCL and FA samples, however, the FA fraction is devoid of the abundant cytosolic protein, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 6B). These data indicate that we are able to selectively preserve adhesion associated proteins, however, this is by no means an exhaustive assessment of the efficacy of the FACS preparation. Following the first proteomic analysis, it may be necessary to further optimize and characterize this process (i.e. non-adhesion associated proteins are identified in the FA fraction). Immunoblots with antibodies against RIIα indicate the presence of PKA regulatory subunit in both the FA and WCL fractions. This finding was replicated with RIIα-BioID2 expressing cells, where overexpressed RIIα-BioID2 was detected in WCL, and to a lesser extent in the FA fraction (data not shown). These results are promising, as the presence of RII subunit in the FA fraction is consistent with the hypothesis that it is recruited to adhesive structures, however, additional testing is required to confirm these initial observations.

*2.5.3. FACS preparation with REF52 cells expressing BioID2/R-subunit constructs*

BioID2 expressing REF52 cells were incubated in biotin containing media for 17-20 hours prior to isolation of adhesive complexes by FACS preparation. We observed that biotin labeled proteins were present in both the WCL and FA fractions (Fig. 6C-D). Western blot analysis with horse radish peroxidase (HRP) conjugated streptavidin reveals

that while the abundance of labeled proteins appeared to be greater in the WCL fraction (as evidenced by the shorter exposure time required), each fraction has a distinct pattern of bands. This suggests that the labeled proteins in the FA fraction are not simply a result of low level of cytosolic contamination in the preparation, but rather, constitute a unique population of labeled species. Interestingly, there appear to be differences in the focal adhesion/cytoskeletal proteins that are labeled by RI versus RII BioID2 fusion constructs, and even, to a lesser extent, between the N and C-terminal constructs.

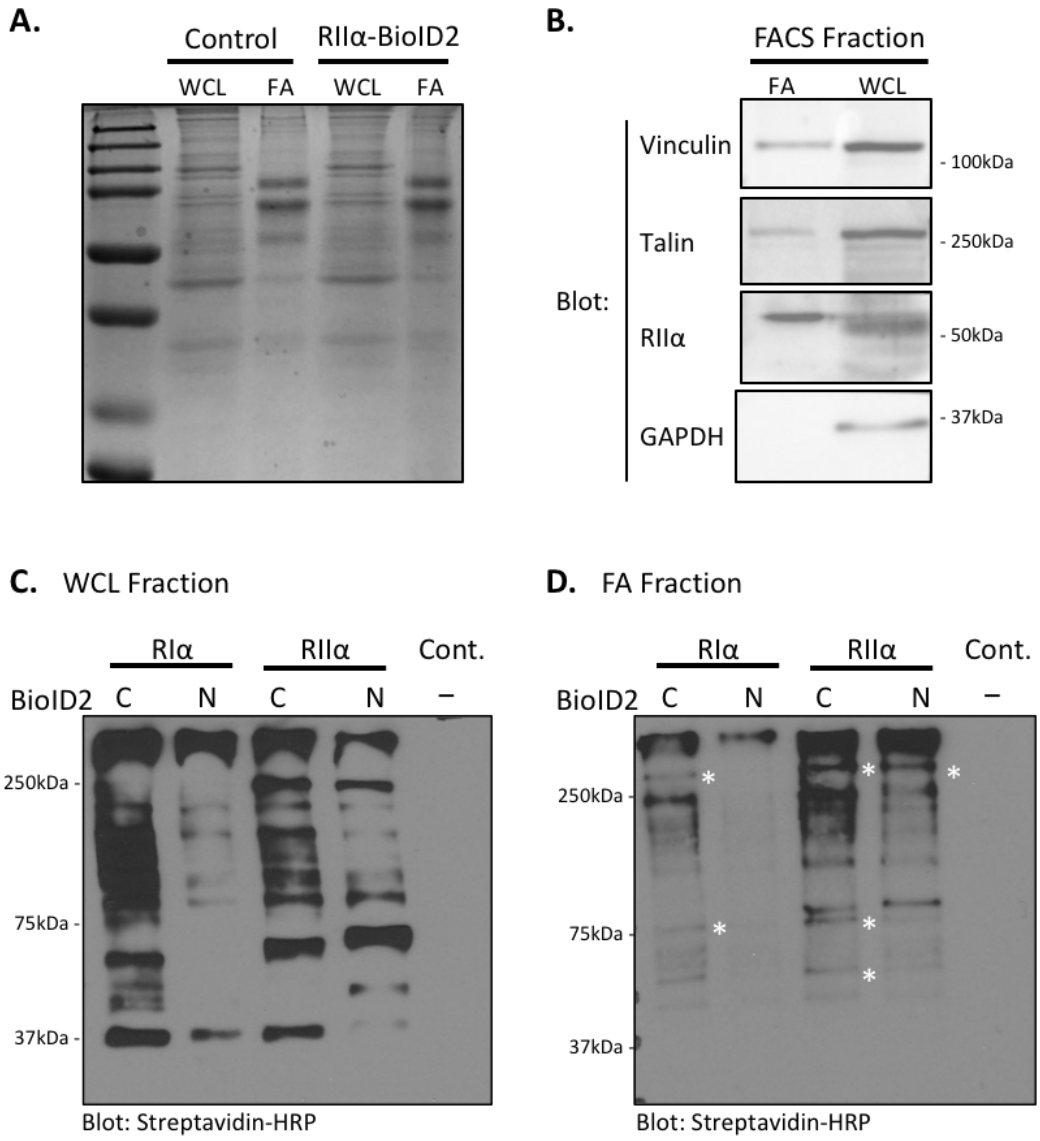


**Figure 6. Characterization of FACS preparation in REF52 cells.**
(**A**) The compositions of the WCL and FA fractions from FACS preparations of non-transfected (Control) or RIIα-BioID2 expressing REF52 cells were compared by Coomassie staining. Equal volumes (20 µl) of WCL and FA fractions were separated by SDS-PAGE and stained with colloidal Coomassie. (**B**) Immunoblots of FA and WCL fractions from a FACS preparation with non-transfected REF52. (**C-D**) Non-transfected (Cont.) and transiently expressing REF52 cells were treated with 50 µM biotin for ~18 hours prior to FACS preparation. Biotin labeled proteins in the WCL (**C**) and FA (**D**) fractions were detected by streptavidin-HRP blot. WCL and FA fraction blots were exposed to film for 5 minutes and overnight, respectively. Asterix in **D** indicate bands that are not present in the corresponding WCL fraction.

### 2.6 Affinity capture of biotin labeled proteins with streptavidin coated magnetic beads

Following acetone precipitation of the FA fraction, labeled proteins are isolated by affinity capture using Dynabead MyOne Streptavidin T1 (Thermo Fisher Scientific) streptavidin-conjugated, magnetic beads. We considered several conditions for the capture, wash, and elution steps (see Supplemental Methods) in order to find the parameters most compatible with our sample composition and the downstream proteomic analysis. The procedure detailed here was informed by the results of preliminary experiments in which biotin labeled proteins were purified from both whole cell extract and the FA fraction of the FACS preparation. It is important to note that this protocol may require further optimization pending the findings of the first, full-scale MS analysis.

### 2.7 Selection of BioID2/R subunit construct for initial, large scale experiments

Given that the leading edge PKA activity required for cell migration is mediated through RII anchoring (McKenzie et al., 2011) and that disruption of RII anchoring impacts focal adhesion dynamics (McKenzie, unpublished), we opted to utilize a RIIα subunit BioID2 construct for the initial execution of the AKAP screen. RIIα-BioID2 and BioID2-RIIα have comparable ligase activity and label proteins in both the FA and WCL fractions, however, we observed that REF52 cells do not express full-length RIIα-I3S,I5S-BioID2 mutant protein that is recognized by the RII specific antibody (Fig. S2C). Interestingly, a band that appears to correspond to the full-length protein is visible in the anti-myc antibody immunoblot of the same samples (Fig. S2B). In light of the fact that the N-terminal construct carrying the same mutation is recognized by the RII antibody, it does not seem

likely that the mutation would impair antibody recognition in the C-terminal fusion. At this time, we have not determined the cause of this discrepancy, however, as we anticipate using the paired 'wild type' and AKAP binding mutant BioID2 constructs in later experiments, we elected to proceed with BioID2-RIIα.

# CHAPTER III: PROCEDURE

## 3.1 Materials

- Dulbecco's Modification of Eagles Medium (DMEM) 4.5 g/L glucose (Corning; 10-013-CV)
- Fetal Bovine Serum (FBS)
- 0.05% Trypsin, 0.53 mM EDTA (Corning; 25-052-Cl)
- Opti-MEM (Gibco; 31985-070)
- Fugene 6 Transfection Reagent (Promega; E2691)
- 1 mM biotin, DMEM (20x stock solution)

  **Note:** sterile filter after addition of biotin. Can be stored for 8 weeks at 4°C. Prepare 50 μM working solution immediately before use by diluting in DMEM and FBS (final concentration 10%)
- Dublbecco's Phosphate Buffered Saline with calcium chloride and magnesium (Complete PBS, Sigma; D1283)
- DSP/DBDPB Crosslinking Solution (0.5 mM dithiobis[succinimidyl propionate] (DSP), 0.05 mM 1,4-di-[3'-(2'-pyridyldithio)-propionamido]butane (DPDPB))

  **Note:** prepare in 1x complete PBS. Warm reagents and complete PBS to 60°C and vortex thoroughly to prevent precipitation of DSP.
- RIPA Buffer (1% NP40, 0.5% sodium deoxycholate (NaDOC), 0.1% SDS. 50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1mM EDTA)
- HALT protease inhibitor cocktail (Thermo Fisher Scientific, 78430)

- DTT reducing buffer (25 mM Tris-HCL pH 7.5, 10 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 100 mM Dithiothreitol (DTT))
- Acetone (Sigma-Aldrich, A4206)
- GlycoBlue Coprecipitant (Fisher Scientific, am9515)
- Non-reducing Laemmli Sample Buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue)
- Modified RIPA (1% NP40, 0.5% sodium deoxycholate, 50 mM Tris pH 7.5, 150 mM sodium chloride)
- Dynabeads MyOne streptavidin T1(Thermo Fisher Scientific, 65601)
- Wash buffer for streptavidin affinity purification (25 mM Tris, 0.5% NP40, 140 mM NaCl, 1% Triton X-100)
- 1x Laemmli Sample Buffer, 4 mM Biotin

  **Note:** use 6 mM biotin stock solution. When preparing stock solution; add 1N NaOH dropwise to increase the solubility of biotin in water.

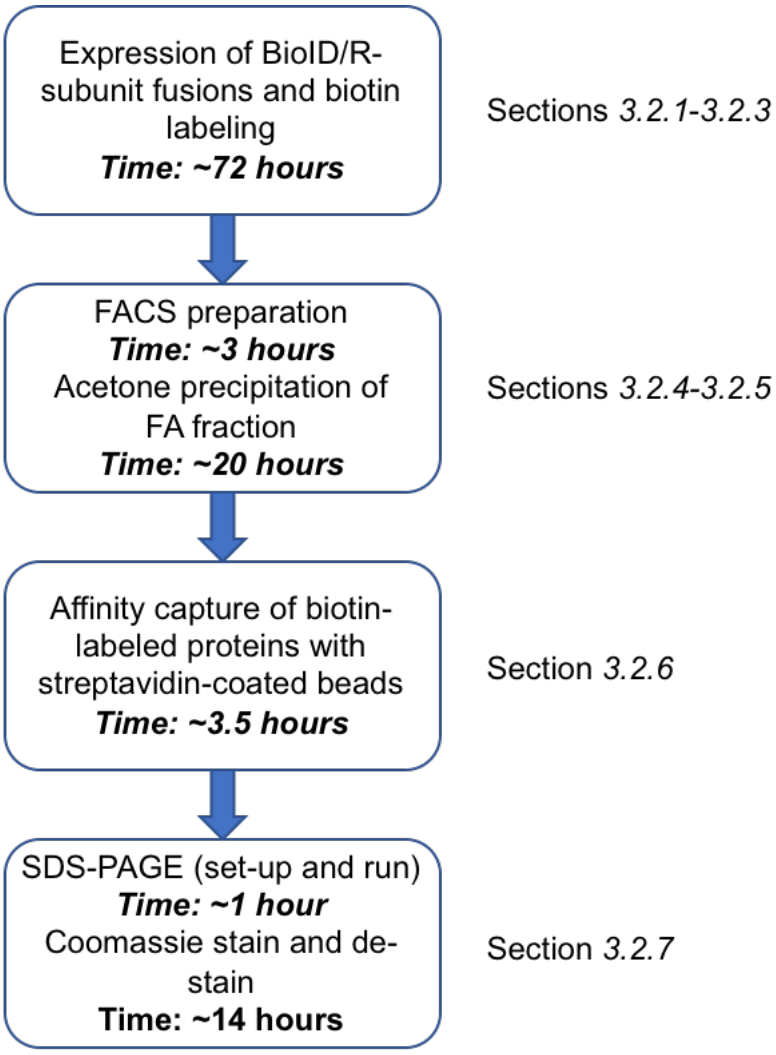


**Figure 7. Overview of Procedure**

## 3.2 Protocol

*3.2.1 Seeding cells*

1) Seed 165,000 REF52 cells per 6 cm tissue culture dish. Prepare enough dishes for each transfection and a control. Use DMEM supplemented with 10% FBS and maintain cells at 37°C in a humidified incubator with 5% $CO_2$.

*3.2.2. Transfection*

**Timing:** 24 hours after seeding

**Note:** cells should be approximately 70% confluent prior to transfection. To scale-up; multiply the following volumes by the number of dishes that will be used for transfection or as controls and prepare a single master transfection or mock solution for each condition.

1) Aliquot 300 µl of Opti-MEM into 2 sterile 1.5 mL microcentrifuge tubes and warm in a 37°C water bath. Label tubes: "BioID2-RIIα" or "Mock".
2) Bring both the FuGENE 6 transfection reagent and purified RIIα-BioID2 plasmid DNA to room temperature.
3) In the biosafety cabinet; vortex FuGENE 6 and add 18 µl to the tube of Opti-MEM labeled "BioID2-RIIα" and vortex for 5 seconds. Add the volume equivalent to 4.5 µg of BioID2-RIIα plasmid to the mixture and vortex for 5 seconds. Let the solution sit, undisturbed for 20 minutes.

***Critical Step:*** add FuGENE 6 directly to the Opti-MEM solution. Do not let the reagent come in contact with the side of the tube.

4) Add the solution from the “BioID2-RIIα” tube dropwise to one of the 6 cm dishes of cells. Swirl gently to mix and label “BioID2-RIIα” before returning the dish to the incubator.
5) Add the solution from the “mock” tube to the second dish and label “Control”.

*3.2.3 Plating cells for FACS preparation*

**Timing:** 24 hours after transfection

1) Prepare DMEM supplemented with 50 μM Biotin and 10% FBS from 20x stock as described above.

**Note:** prepare 10 mL of solution per dish

2) Warm biotin containing media and biotin-free DMEM supplemented with 10% FBS in a 37°C water bath.

**Note:** warm ~ 4 mL of DMEM + 10% FBS per 6 cm dish

3) Rinse one of the dishes (“BioID2-RIIα” or “Control”) with 5 mL of sterile, room temperature 1x complete PBS.
4) Add 1 mL of Trypsin (0.05% Trypsin/0.53 mM EDTA) and return the dish to the incubator for 5 minutes, or until all cells have detached from the plate.
5) Resuspend trypsinized cells in 4 mL of DMEM/10%FBS and move the cell solution to a 15 mL conical tube.
6) Centrifuge cells at 50x g for 5 minutes at room temperature. During centrifugation, label a 10 cm tissue culture dish as either “BioID2-RIIα” or “Control” as appropriate.

7) Following centrifugation, carefully aspirate the excess media from the cell pellet and *gently*, but *thoroughly* resuspend the cell pellet in 10 mL of DMEM/50 μM biotin/10% FBS and transfer the entire volume to the labeled 10 cm dish.
8) Repeat steps 3 – 7 above with the second dish of cells.

*3.2.4 FACS preparation*

**Timing:** begin 17-20 hours after replating in biotin containing media

**Note:** cells should be > 70% confluent at the start of this procedure. Prepare crosslinking and elution solutions as described above prior to beginning FACS protocol

1) Transfer dishes from the incubator to the bench-top and gently wash each 10 cm dish 1x with ~10 mL of cold, 1x complete PBS.
2) Aspirate the majority of the 1x complete PBS from each dish and then, using the lids, prop the dishes at an angle for approximately 1-2 minutes to collect residual liquid. Aspirate pooled liquid.
3) Add 5 mL of DSP/DPDPB crosslinking solution to each dish and incubate for **5 minutes**.
4) Aspirate the crosslinking solution and immediately add 5 mL 1M TRIS pH 7.5 to quench the reaction.
5) Aspirate the TRIS solution and prop dishes at an angle for approximately 1-2 minutes. Aspirate remaining solution.
6) Transfer dishes to ice and add 500 μl of RIPA buffer containing protease inhibitors. Tilt the dish in all directions until the entire surface is covered by lysis buffer.

Incubate on ice for **30 minutes** - tilting the dishes every 5-10 minutes to ensure full coverage.

7) Prop the dishes at an angle (using the lid or the side of the ice bucket) and allow lysate to pool for 1-2 minutes before transferring the lysate to a pre-chilled tube. This is the *"whole cell lysate"* (WCL) fraction. Ensure tubes are appropriately labeled and store at -80°C.

***Critical step:*** do not scrape the dish to collect lysate at this point

**Note:** when performing replicates; pool lysates and then store in 500 µl aliquots.

8) Keep dishes on ice and add ~2 mL cold, 1x complete PBS to each dish to prevent drying
9) Fill the reservoir of the 'waterpik' (Conair Interplak, or similar) with cold, 1x complete PBS and ensure that the instrument is set properly (Interplak setting 7; ~30 psi).
10) Hold the dish at an angle (~90-110°) over a collection vessel and position the pik approximately **1 cm** from the surface with the stream perpendicular to the dish.
    i. Starting at the very top of the dish, slowly move the stream from side-to-side while gradually moving down toward the bottom of the dish.
    ii. Upon reaching the opposite side, change directions and repeat the side-to-side movement back up the dish.
    iii. Move the stream along the perimeter (focusing on the area where the bottom and the sidewall of the dish meet)

**Note:** The PBS that collects in the vessel under the washing station can be used to refill the waterpik reservoir.

11) Dry the bottom of the dish with a lint-free wipe or paper towel and visually inspect the entire surface (by 10X objective).
    i. If nuclei are present: mark any regions of the dish where nuclei are visible and repeat washing procedure focusing the stream on the indicated areas.
    ii. Repeat visual inspection

**Note:** to find the correct focal plane; begin by focusing on the receding line of PBS as it pools on the dish. Focusing up and down at this line will help you locate the bottom of the dish. Residual nuclei are likely to be located near the perimeter of the dish. Cytoskeletal fibers may also be visible; do not perform additional washes to remove visible cytoskeleton.

12) When all nuclei have been cleared - wash the dish with 5 mL fresh, complete PBS. Aspirate and tilt dish to collect as much of the liquid as possible.

    Note: at this stage a small amount of PBS tends to pool in the center of the dish. Aspirate as much as possible, but it is acceptable to leave a small amount.

13) Add 500 µl DTT elution buffer to each dish and tilt the dish to spread evenly

    Note: it may be necessary to use a plastic policeman to *gently* spread the elution buffer over the surface of the dish. If so, set the policeman aside for use in Step 28 below.

14) Replace the lids and transfer the dishes to a 60°C oven for **10 minutes**.

15) Holding the dish at an angle, use a plastic policeman to scrape the entire surface of the dish toward the bottom edge to collect the solution. Transfer this solution to a clean 1.5 mL microcentrifuge tube and label appropriately. This is the *"focal adhesion"* (FA) fraction.

***Critical step:*** scrape dish thoroughly to ensure maximum recovery (dish should appear dry everywhere except where solution has collected). The volume recovered at this step should be between 350-400 µl (record the actual volumes for use in *3.2.5* Step 1 below).

**Note:** when performing replicates, pool the FA fractions at this stage

16) Place the tubes containing the FA fraction in a 60°C water bath for **50 minutes**.

*3.2.5 Acetone precipitation of focal adhesion fraction*

1) Remove the tubes from the water bath and add a volume of ice cold acetone that is 4X the volume of the recovered FA fraction (as noted in Step 28).

**Note:** glycol blue can be added at this stage to improve the visibility of the protein pellet. If FA fraction was pooled in the previous step, it may be necessary to re-aliquot the sample to accommodate the maximum volume of the 1.5 mL microcentrifuge tube.

2) Incubate at -20°C overnight.

**Note:** samples can be stored at -20°C for 24-48 hours at this stage

3) Centrifuge samples at ≥ 13,000x g for 15-30 minutes at 4°C
4) Tilt the 1.5 mL microcentrifuge tube at a 45° angle with the protein pellet located at the top and *carefully* aspirate the supernatant, tracking the pipet tip along the bottom side of the tube.

5) Leave the lids open and allow the pellets to dry on the bench-top for **15-20** minute.

***Critical Step:*** do not allow the pellets to over-dry. Pellets should be just visible and slightly translucent.

6) Resuspend each pellet in 20 µl of non-reducing Laemmli sample buffer. Flick tube or carefully triturate to mix (avoiding bubbles).

**Note:** if fragments of the pellet fail to go into solution, add additional non-reducing LSB in 5 µl increments.

7) Store samples at -20 – -80°C.

*3.2.6 Affinity capture of biotin labeled proteins*

1) Vortex Dynabeads MyOne Streptavidin T1 magnetic beads for 30 seconds.
2) Aliquot 50 µl of bead suspension into each 1.5 mL microcentrifuge tube (1 tube per condition).
3) Wash beads with 1 mL of modified RIPA buffer.
4) Place tubes on magnetic stand for 1 minute and then remove and discard the supernatant.
5) Resuspend beads in 50 µl of modified RIPA buffer.
6) Add thawed FA fraction sample to bead suspension and incubate for 2 hours at 4°C with gentle rotation.
7) Place tubes on magnetic stand for 1 minute and remove the supernatant.

**Note:** supernatant can be collected and stored at -80°C for additional testing if necessary.

8) Add 1 mL of wash buffer.

9) Place tubes on magnetic stand for 1 minute and then remove and discard the supernatant.

10) Repeat washing steps 3 more times using 500 µl of wash buffer for each wash.

**Note:** there is no volume restriction for wash steps and volumes of each wash can be adjusted as appropriate.

11) Following removal of the final wash, add 25-50 µl of Elution buffer (1X LSB, 4 mM biotin) and boil for **3 minutes**.

**Critical step:** do not boil for longer than 5 minutes. Prolonged exposure to high temperatures can lead to aggregation of the denatured streptavidin monomers released from the beads.

12) Allow solution to sit at room temperature for 15 minutes and then place tubes on the magnetic stand for 1 minute and *collect* the eluate.

**Note:** (optional) retain 5% of final sample for additional testing before proceeding to the next step.

*3.2.7 Separation by SDS-PAGE and Coomassie staining*

1) Cast a 1.5 mm 10% separating poly acrylamide gel with a 3.5% stacking gel

**Note:** The reduced percent polyacrylamide in the stacking gel allows for complete transfer of the higher molecular weight species to the separating gel during the abbreviated run time.

2) Load entire volume of each sample (BioID2-RIIα and control) along with a pre-stained protein ladder and run at 100 V until the ladder enters the separating

portion of the gel. Run at 150V for approximately 9 minutes, or until there is 1 cm of separation between the top band and bottom band of the ladder.

**Note:** it is important to run the samples far enough so that the free streptavidin (~15kDa) can be easily excised, but the separation should be minimized as much as possible to avoid additional processing steps before MS analysis.

3) Separate glass plates and remove gel.
4) Cover gel with colloidal Coomassie blue stain and incubate at room temperature with agitation for 1 – 2 hours.
5) De-stain gel in 10% acetic acid at room temperature with agitation for up to 12 hours.

**Note:** the samples are now ready to be submitted for proteomic analysis

# CHAPTER IV: SUPPLEMENTAL INFORMATION

## 4.1 Supplemental Methods

### *4.1.1 Plasmids*

The sequences for PKA RIIα and RIα were amplified from pcDNA3-mouse PKA-RIIalpha-mEGFP (Addgene plasmid #45527) and pcDNA3-mouse PKA-RIIalpha-mEGFP (Addgene plasmid #45525) using PfuUltra II Fusion HS DNA Polymerase (Agilent, 600670). The primers used for amplification were designed with 5' and 3' overhangs of 15-20 nucleotides complementary to the appropriate BioID2 vector (Table S1). Similarly, the BioID2 vector sequences were amplified from either MCS-13X Linker-BioID2-HA (Addgene plasmid #80899) or Myc-BioID2-13X Linker-MCS (Addgene plasmid # 92308) with overhangs complementary to the RIα or RIIα sequence (Table S1). PCR products (1 μg of each) were digested with Dpn I (New England Biolabs) for 30 minutes at 37℃ and then at 80℃ for 20 minutes to heat inactivate the Dpn 1 enzyme. The R subunit fragments were ligated with BioID2 vectors at a 3:1 molar ratio with Gibson assembly master mix (New England Biolabs, E5510S) for 15 minutes at 50℃. DH5α competent Escherichia coli cells (New England Biolabs, C2988J) were transformed with 2 μl of each assembly reaction according to the manufacturer's recommended procedure and grown on ampicillin containing agar plates overnight at 37℃. Selected colonies were grown in ampicillin containing LB broth overnight and then purified using the Monarch Plasmid Miniprep kit (New England Biolabs, T1010S). Purified clones and BioID2 parent vectors were digested with BamHI (New England Biolabs) for 30 minutes at 37℃ to

confirm insertion of the R subunit fragments. Select positive clones were then sequenced using standard primers (CMV_F and BGH_R; Eurofins).

BioID2/RIIα(I3S, I5S) and RIα(C37H) AKAP binding mutant constructs were generated by site directed mutagenesis of the wild type plasmids using the QuickChange kit (Agilent, 200518) with plasmids: gatgagccacAGCcagAGCcccgcggggc and gccccgcgggGCTctgGCTgtggctcatc for RIIα(I3S, I5S), and ccatcgtgcagctgCACactacgcggcccgagag and ctctcgggccgcgtagtGTGcagctgcacgatgg for RIα(C37H).

*4.1.2 Immunoblotting*

Cell lysates were mixed with 5x Laemmli Sample Buffer (LSB; 10% SDS, 50% glycerol, 25% β-mercaptoethanol, 3 M Tris, 0.05% bromophenol blue) and boiled for 10 minutes and then separated via SDS-PAGE (10% polyacrylamide) in Tris-glycine/SDS running buffer (25 mM Tris, 190 mM glycine, 0.1% sodium dodecyl sulfate). Proteins were transferred to a 0.2 μM Polyvinylidene difluoride membrane (PVDF; unless otherwise noted) for 90 minutes at 50 V, or 60 minutes at 80 V in cold transfer buffer (0.5X running buffer, 20% methanol) and blocked in either 5% bovine serum albumin (BSA) or 5% non-fat dry milk (NFDM) in Tris-buffered saline, 0.1% Tween 20 (TBST) at 4℃ overnight (Table S2). Membranes were then incubated with primary antibody diluted in the appropriate blocking solution as indicated (Table S2) at 4℃ overnight. Following removal of the primary antibody solution, membranes were washed 5 times for 5 minutes each in TBST. Membranes were then incubated for 1 hour with horseradish peroxidase (HRP)

conjugated, mouse or rabbit secondary antibodies, diluted 1:5000 in the appropriate block solution. Following removal of the secondary antibody solution, membranes were washed again in TBST, incubated with Supersignal™ West Pico Chemiluminescent Substrate (Thermo Scientific, PI34080) for 5 minutes and either exposed to film or detected using the Amersham Imager 600 system.

Aliquots of WCL fraction samples from a FACS preparation with transfected REF52 cells were used for the initial characterization of BioID2 fusion proteins (Fig. 4C-D). Lysates from HEK293 cells transfected with wild type or mutant BioID2 constructs were used for comparison of endogenous/overexpressed RIIα (Fig. 4E), characterization of RIIα I3S,I5S BioID2 fusions (Fig. S2), and streptavidin-bead affinity purification (Fig. 5). HEK293 cells were lysed 48 hours after transfection using modified RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 50 mM Tris pH 7.5, 150 mM sodium chloride) with HALT protease inhibitor cocktail (Thermo Fisher Scientific, 78430). Lysates were cleared by centrifugation at ≥ 13,000xg for 15 minutes at 4℃. The protein concentration of REF52 WCL samples and HEK293 lysates was determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, 23225). For characterization of stable REF52 cell lines, cells were lysed in 1X LSB, heated at 60℃ for 6-12 hours, and boiled for 10 minutes (Fig. S1).

*4.1.3 Biotin labeling and streptavidin-HRP blotting*

Cells transfected with BioID2 constructs were incubated in DMEM supplemented with 50 μM biotin and 10% FBS for 17-24 hours approximately 24 hours post transfection (note: labeling was detected at as early as 6 hours after the addition of biotin in REF52

cells). Samples from FACS preparations or from cells lysed in 1X LSB were prepared as described above. Proteins were transferred to 0.2 μm nitrocellulose membranes (Note: PVDF membranes were also tested and appear suitable for this application, however, nitrocellulose performed better when compared side-by-side). Membranes were blocked in 5% BSA, TBST for 30 minutes at room temperature and then incubated in a 1:20,000 dilution of streptavidin-HRP (Thermo Fisher Scientific, 21126) in 5% BSA, TBST for 1 hour at room temperature (Note: 1:40,000 dilution was not sufficient for detection of labeled protein after 6-hour biotin exposure, however, this dilution was not tested on samples with longer biotin incubation times). Following removal of streptavidin-HRP solution, membranes were washed 5 times for 5 minutes each in TBST and developed as described above.

### *4.1.4 Quenching of HRP*

Membranes that had been treated previously with either streptavidin-HRP or HRP conjugated secondary antibodies were incubated in a solution of 1% sodium azide and 1.5% hydrogen peroxide for 10 minutes at room temperature to quench HRP enzyme activity. Membranes were then incubated in 10% adult bovine serum, TBS-T for 30 minutes at room temperature before being moved to 5% BSA, TBS-T for 1 hour at room temperature (can be stored overnight at 4C) before addition of primary antibody

*4.1.5 Determination of FA fraction protein concentration*

The protein concentration of the WCL fraction was first determined by BCA assay and then equal volumes of WCL (prepared as described for immunoblotting) and FA fraction samples were separated by SDS-PAGE (10% acrylamide). Gels were stained with a colloidal Coomassie blue solution for 1-2 hours and then destained with 10% acetic acid for up to 12 hours. The relative ratio of FA fraction protein to WCL protein was determined by densitometry using the FIJI Gel Analyzer function (Image J). Using this ratio, the protein concentration of the FA fraction was calculated from the previously determined concentration of the WCL fraction. The total yield of the FACS preparation was calculated by multiplying the FA or WCL concentration by the sample volume (for a single dish or pooled samples; Table S3).

*4.1.6 Affinity capture of biotin labeled proteins from cell extracts and alternative methods*

HEK 293 cells transiently expressing BioID2/R-subunit fusion proteins and non-transfected control cells were washed one time with complete PBS and then lysed in modified RIPA buffer containing HALT protease inhibitor cocktail. The lysates were cleared as described above, and then incubated with Dynabeads MyOne streptavidin T1 beads as outline in section *3.2.6*.

*Alternative capture conditions*

In preliminary experiments we resuspended the acetone precipitated FA fraction in reducing LSB. After several rounds of troubleshooting and conversations with the Dynabead manufacturer's technical support team, we could not rule out the possibility that the harsh reducing agents present in the LSB are incompatible with the magnetic beads. Though we did not exhaustively test this hypothesis, we opted to proceed using non-reducing sample buffer. Another option that was considered but not fully evaluated is the dilution of the FA fraction with a buffer that would bring the composition of the sample closer to that of a typical lysis buffer known to be compatible with the magnetic beads. For example; the LSB containing sample (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris) can be 10-fold diluted with a buffer containing Tris, Triton X-100, and NaCl to achieve a final composition of 0.2% SDS, 1% glycerol, 0.5% β-mercaptoethanol, 50 mM Tris, 1% Triton X-100. This is very near to the composition of the lysis buffer specified for this application in a protocol provided by the laboratory of Dr. Scott Gerber at Dartmouth College (personal communication).

*Alternative wash conditions*

The wash buffer (25 mM Tris, 0.5% NP40, 140 mM NaCl, 1% Triton X-100) utilized in this protocol was recommended by our colleagues in the lab of Dr. Jason Botten at the University of Vermont (Ziegler et al., 2017). As we have not yet fully evaluated the efficacy of this wash buffer in our experimental system, if the results of the first full scale trial indicate that it is not stringent enough, the above-mentioned protocol from the Gerber

laboratory utilizes a different wash method. In this protocol, beads are washed with a detergent containing buffer (0.2% SDS, 1% NP-40, 1xPBS) followed by a high salt buffer (0.2% SDS, 1% NP-40, 400 mM NaCl, 1xPBS).

*Alternative elution conditions*

Biotinylated proteins can be eluted from magnetic beads by incubation with 1xLSB at 100℃ for 3-5 minutes. We found that this method resulted in significant stripping of streptavidin monomer from the magnetic beads (visible after Coomassie staining as a prominent band at ~15 kDa). To avoid confusion in downstream analyses as a result of contamination from the abundant streptavidin monomer, we attempted to elute biotinylated proteins from the beads with 1xLSB supplemented with 6M urea at 50℃ for 1 hour (note: urea should not interfere with MS analysis as long as the samples are not heated above 60℃). While this method did prevent the stripping of streptavidin from the beads, MS analysis of samples treated with 6M urea, 1xLSB did show subtle differences in the peptide profile when compared to samples treated with 1xLSB alone. For this reason, we opted to avoid adding urea to the elution buffer. It has also been reported in the literature that boiling with 1xLSB is not sufficient for full recovery of biotin labeled proteins, and that addition of excess biotin can increase the yield by competing off bound proteins (Cheah and Yamada, 2017; Rybak et al., 2004). Based on recommendations from the Gerber laboratory, we endeavored to improve the recovery of biotinylated proteins by eluting in 1xLSB with 4 mM biotin by incubating at room temperature for 15 minutes and then at 85℃ for 15 minutes. This resulted in the formation of a green precipitate that was weakly

magnetic and may have been chelated iron from the beads. The Gerber lab utilizes streptavidin conjugated agarose beads for the purification of biotin labeled proteins, and it is not clear at this time why this elution method appears to be incompatible with magnetic beads.

## 4.2 Supplemental Tables and Figures

**Table S 1. Primers used in the cloning of BioID2/R-subunit constructs**

| Construct | Vector | Primer Sequence (5'→ 3') |
|---|---|---|
| RIIα-BioID2 | RIIα | F: gctggctagcgcttaaggccaccatgagccacatccagatcccc |
| | | R: cctccaccggatccgaattcctgcccggggtcca |
| | BioID2-HA | F: atctgatggaccccgggcaggaattcggatccggtggagg |
| | | R: atctggatgtggctcatggtggccttaagcgctagccag |
| BioID2-RIIα | RIIα | F: agcagcgcggaggcggtggatcggcgatgagccacatccagatccc |
| | | R: ggcacagtcgaggctgatcactgcccggggtccat |
| | Myc-BioID2 | F: atctgatggaccccgggcagtgatcagcctcgactgtgcctt |
| | | R: gggatctggatgtggctcatcgccgatccaccgcc |
| RIα-BioID2 | RIα | F: gctggctagcgcttaaggccaccatggcgtctggcagtatggc |
| | | R: ccgcctccaccggatccgaagacggacagggacacgaagc |
| | BioID2-HA | F: gcttcgtgtccctgtccgtcttcggatccggtggaggcg |
| | | R: atactgccagacgccatggtggccttaagcgctagccag |
| BioID2-RIα | RIα | F: gcggaggcggtggatcggcgatggcgtctggcagtatgg |
| | | R: ggcacagtcgaggctgatcagacggacagggacacgaagc |
| | Myc-BioID2 | F: gcttcgtgtccctgtccgtctgatcagcctcgactgtgcc |
| | | R: gccatactgccagacgccatcgccgatccaccgcc |

**Table S 2. Antibodies used for Immunoblotting**

| Antibody | Species | Manufacturer, item # | Dilution | Blocking solution |
|---|---|---|---|---|
| myc | mouse | Cell Signaling, CS2276 | 1:1000 | 5% NFDM, TBS-T |
| HA | rabbit | Cell Signaling, CS3724 | 1:1000 | 5% BSA, TBS-T |
| RII | mouse | BD, Bioscience, 612242 | 1:1000 | 5% BSA, TBS-T |
| Talin | mouse | Sigma-Aldrich, T3287 | 1:1000 | 5% BSA, TBS-T |
| Vinculin | mouse | Sigma-Aldrich, V9131 | 1:200 | 5% BSA, TBS-T |
| GAPDH | rabbit | Cell Signaling, 2118 | 1:100 | 5% BSA, TBS-T |

**Table S 3. Example Yields from FACS preparation with REF52 cells**

| Cell confluence | Protein in WCL fraction (per dish) | Protein in FA fraction (per dish) |
|---|---|---|
| 100% | 210-240 µg | 24 µg |
| 70% | 99 µg | 7 µg |

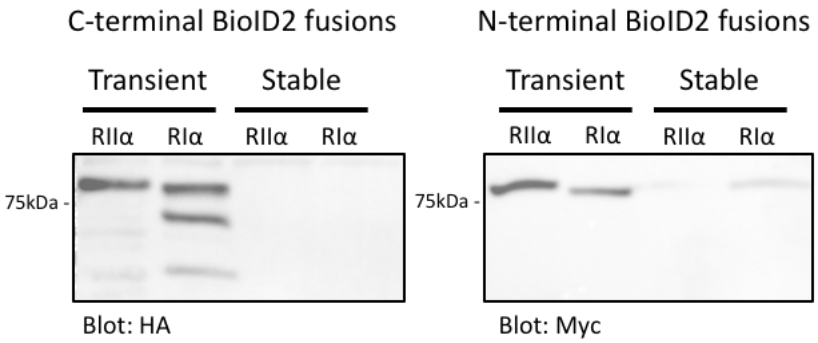


**Figure S 1. Expression of BioID2/R-subunit fusions in transiently transfected and G418 resistant REF52 cells.**

Equal amounts of lysate from transiently expressing (Transient) or G418 resistant (Stable) REF52 cells were separated by SDS-PAGE and immunoblotted with antibodies against the appropriate epitope tags. The expression of the C, and N terminal BioID2 fusions was assessed by immunoblot using antibody against HA (left) and myc (right) respectively.

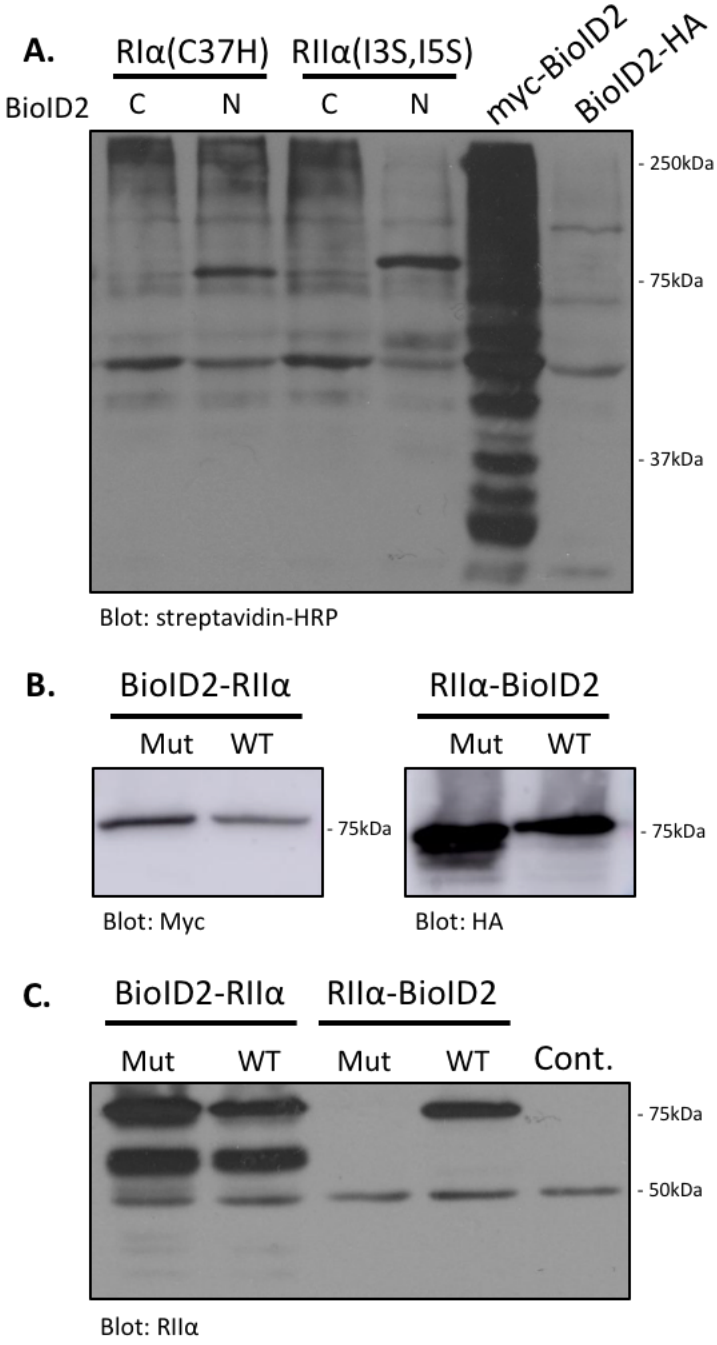


**Figure S 2. Characterization of biotin ligase activity and expression of AKAP binding mutant BioID2/R-subunit fusions.**

(**A**) REF52 cells expressing RIα(C57H) BioID2 fusions, RIIα(I3S,I5S) BioID2 fusions, or untargeted BioID2 constructs were incubated with 50 μM biotin for 20 hours prior to lysis. Lysates probed with streptavidin-HRP to assess biotin ligase activity. (**B-C**) Lysates from transiently expressing HEK 293 cells were probed with antibodies against the myc or HA epitope tags (**B**) to detect C (left) and N-terminal (right) BioID2 fusions respectively or antibody against RIIα to detect endogenous and BioID2 fusion protein (**C**).

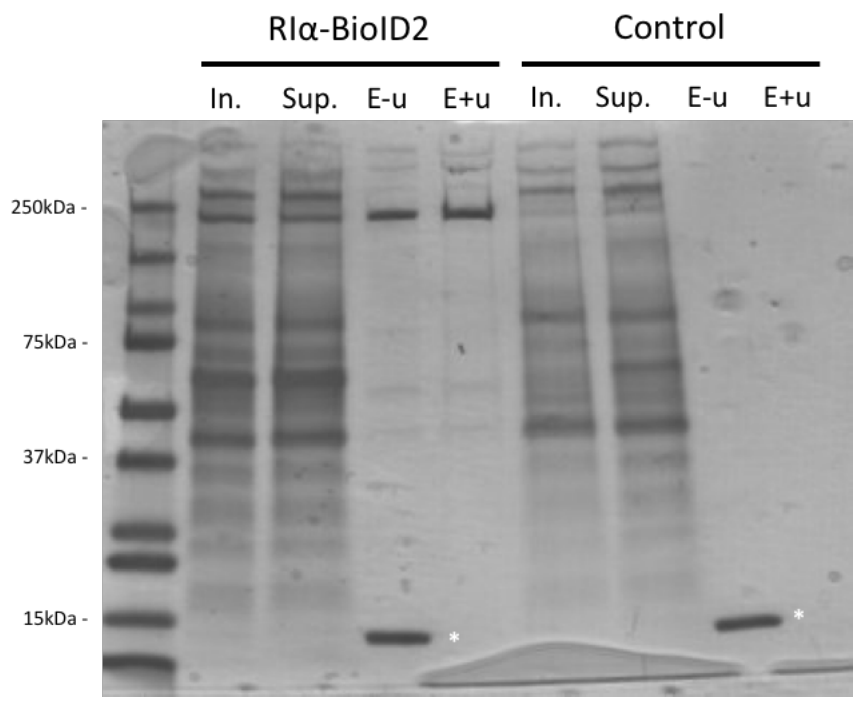


**Figure S 3. Comparison of elution conditions for streptavidin affinity purification.** Non-transfected (Control) and RIα-BioID2 expressing REF52 cells were incubated with 50 µM biotin for X hours and lysed in modified RIPA buffer. Streptavidin affinity purification was performed using 126 µg and 90 µg of RIα-BioID2 and control lysate, respectively. Each sample was divided into two parts during the final wash step; one fraction was eluted by boiling in 1x LSB (E-u) and the other in 1x LSB, 6M urea (E+u). Equal amounts of protein from the whole cell extract (In.) and supernatant (sup) from the affinity capture step, and the entire volume from each elution fraction were analyzed. Asterix indicate streptavidin monomer.

## CONTRIBUTIONS

I gratefully acknowledge Kathryn Svec for creation of the BioID2/R-subunit AKAP binding mutant constructs; Zoe Edmunds for western blots related to FA and WCL fraction characterization, generation of stable cell lines, and assistance with FACS preparations; John Patterson for assistance with FACS preparations; Dr. Kyle Roux for providing the MCS-13X linker-BioID2-HA and Myc-BioID2-13X linker-MCS plasmids; Dr. Scott Gerber for guidance in the purification of biotin-labeled proteins; Catrina Hood and Dr. Ying Wai Lam of the Vermont Genetics Network Proteomics Facility for their assistance planning and troubleshooting proteomic analysis; and former graduate students Laura Director and Andrew McKenzie for providing the foundation for this work.

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